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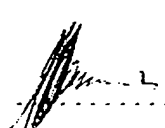
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DATED this 23rd

day of March

19 92

  
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- (57) Claim

1. A process for inactivating viruses in blood and blood products, comprising: adding phenothiazine dyes to the solutions or suspensions to be treated and subsequently irradiating said phenothiazine-dye containing solutions or suspensions with visible light in the range of the absorption peak of the respective dye, whereafter the blood or blood products may be passed over adsorbing agents for removal of the dyes, characterized in that the phenothiazine dyes are used at a concentration of from 0.1 to 2  $\mu$ M and irradiation is effected directly in transparent containers, such as blood bags, used for collecting and storing blood.

6. The process as claimed in any one of the claims 1 to 5, characterized in that said process is carried out using two containers suitable for collecting blood, such as blood bags, with a separating column interposed between said containers, and containing the adsorbing agent for the phenothiazine dyes.

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<b>(51) Internationale Patentklassifikation <sup>5</sup> :</b>  <b>A01N 1/02</b>	<b>A1</b>	<b>(11) Internationale Veröffentlichungsnummer:</b> <b>WO 91/03933</b>  <b>(43) Internationales Veröffentlichungsdatum:</b> 4. April 1991 (04.04.91)
<b>(21) Internationales Aktenzeichen:</b> PCT/DE90/00691  <b>(22) Internationales Anmeldedatum:</b> 8. September 1990 (08.09.90)  <b>(30) Prioritätsdaten:</b> P 39 30 510.4      13. September 1989 (13.09.89) DE  <b>(71) Anmelder (für alle Bestimmungsstaaten ausser US):</b> BLUTSPENDEDIENST DER LANDESVERBÄNDE DES DEUTSCHEN ROTEN KREUZES NIEDERSACHSEN, OLDENBURG UND BREMEN G.G.M.B.H. [DE/DE]; Eldagsener Str. 38, D-3257 Springe 1 (DE).  <b>(72) Erfinder: und</b> <b>(75) Erfinder/Anmelder (nur für US):</b> MOHR, Harald [DE/DE]; Rühmkorffstr. 11, D-3000 Hannover 1 (DE). LAM-BRECHT, Bernd [DE/DE]; Marienstraße 1, D-3257 Springe 4 (DE).	<b>(74) Anwalt:</b> SCHUPFNER, Gerhard, D.; Müller, Schupfner & Gauger, Karlstraße 5, D-2110 Buchholz (DE).  <b>(81) Bestimmungsstaaten:</b> AT (europäisches Patent), AU, BE (europäisches Patent), BF (OAPI Patent), BG, BJ (OAPI Patent), BR, CA, CF (OAPI Patent), CG (OAPI Patent), CH (europäisches Patent), CM (OAPI Patent), DE (europäisches Patent)*, DK (europäisches Patent), ES (europäisches Patent), FI, FR (europäisches Patent), GA (OAPI Patent), GB (europäisches Patent), HU, IT (europäisches Patent), JP, KP, KR, LK, LU (europäisches Patent), MC, MG, ML (OAPI Patent), MR (OAPI Patent), MW, NL (europäisches Patent), NO, RO, SD, SE (europäisches Patent), SN (OAPI Patent), SU, TD (OAPI Patent), TG (OAPI Patent), US.  <b>Veröffentlicht</b> <i>Mit internationalem Recherchenbericht.</i>  <div style="font-size: 2em; font-weight: bold; text-align: center;">635068</div>	
<b>(54) Title:</b> PROCESS FOR INACTIVATING VIRUSES IN BLOOD AND BLOOD PRODUCTS  <b>(54) Bezeichnung:</b> VERFAHREN ZUR INAKTIVIERUNG VON VIREN IN BLUT UND BLUTPRODUKTEN  <b>(57) Abstract</b>  The invention relates to a process for inactivating viruses in blood and blood products in which phenothiazine dyes are added to the solutions or suspensions, which are then irradiated with light. The use of a very small concentration of phenothiazine dyes prevents any adverse effects on the plasma proteins. Inactivation is effected by the immediate irradiation of the blood sachet. After irradiation the dyes can be separated from the blood again by passing the blood over adsorbing agents.  <b>(57) Zusammenfassung</b>  Die Erfindung betrifft ein Verfahren zur Inaktivierung von Viren in Blut und Blutprodukten, bei dem die zu behandelnden Lösungen bzw. Suspensionen mit Phenothiazinfarbstoffen versetzt und anschließend mit Licht bestrahlt werden. Durch die Verwendung einer sehr geringen Konzentration an Phenothiazinfarbstoffen werden schädigende Einwirkungen auf die Plasmaproteine ausgeschlossen. Die Inaktivierung erfolgt durch unmittelbare Bestrahlung der Blutbeutel. Nach der Bestrahlung lassen sich die Farbstoffe aus dem Blut wieder abtrennen. Hierzu wird das Blut über Adsorptionsmittel geleitet.		

PROCESS FOR INACTIVATING VIRUSES IN BLOOD  
AND BLOOD PRODUCTS

5 Specification:

The invention is directed to a process for inactivating viruses in blood and blood products, comprising: adding phenothiazine dyes to the solutions or suspensions to be treated and subsequently irradiating said phenothiazine dye-containing solutions or suspensions with visible light in the range of the absorption peak of the respective dye, whereafter the blood or blood products may be passed over adsorbing agents for removal of the dyes.

15

It is known that photodynamic substances in combination with visible light or UV-light may have a virus inactivating effect. This is due to the affinity of these substances to external virus structures or to viral nucleic acid. Both facts apply to phenothiazine dyes. They react with the membrane structures of enveloped viruses and damage the same irreversibly under the action of light, whereby the virus loses its infectiousness (cf. Snipes, W. et al., 1979, Photochem. and Photobiol. 29, 785-790).

25

However, photodynamic substances also interact with viral RNA or DNA, especially with the guanine residues of these nucleic acids. When a dye/nucleic acid-complex has been formed it is stimulated by light energy so that denaturation of the nucleic acid and finally strand breakages result. Also, phenothiazine dyes induce the conversion of molecular oxygen to oxygen radicals which are highly reactive and may have various virucidal effects (cf. Hiatt, C.W., 1972, in: Concepts in Radiation Cell Biology, pp.57-89, Academic Press, New York; Oh Uigin et al., 1987, Nucl. Acid. Res. 15, 7411-7427).

35



1 In contrast to other photodynamic dyes for virus inactivation,  
phenothiazine dyes such as methylene blue, neutral red and  
toluidine blue are of special interest because they can inacti-  
vate a number of viruses already in combination with visible  
5 light and, under certain conditions, even viruses that do not  
possess a lipid envelope, such as adenovirus.

In addition to that, methylene blue (MB) and toluidine blue  
(TB) for instance are themselves being used therapeutically.  
10 among other uses also as antidotes to carbon-monoxide poisoning  
and in long-term therapy of psychotic diseases. In this connec-  
tion quantities of MB or TB much higher than those required  
for virus inactivation are used (1 to 2 mg/kg body weight)  
without any significant side effects. The low toxicities of MB  
15 and TB are also substantiated by data obtained from animal ex-  
periments.

However, since 1955 those of skill in the art have assumed that  
dye concentrations, especially in the case of toluidine blue,  
20 of less than 2.5  $\mu\text{M}$  have only an insufficient virus inactivat-  
ing effect (cf. F. Heinmets et al. 1955, Joint Report with the  
Naval Medical Research Institute, Walter Reed Army Institute  
of Research, U.S.A.).

25 In the previously described investigations of virus inactiva-  
tion with phenothiazine dyes the dye concentrations are between  
10  $\mu\text{M}$  and 100  $\mu\text{M}$  (Chang and Weinstein, 1975, Photodynamic Inac-  
tivation of Herpes-virus Hominis by Methylene Blue (38524),  
Proceedings of the Society for Experimental Biology and Medi-  
cine, 148:291-293; Yen and Simon, 1978, Photosensitization of  
30 Herpes Simplex Virus Type 1 with Neutral Red, J. gen. Virol.,  
41:273-281). But at these concentrations there arises the draw-  
back that not only viruses may be inactivated but also plasma  
proteins, such as the coagulation factors. This is one of the  
35 reasons why phenothiazine dyes have so far not achieved any  
significance in the inactivation of viruses in blood and blood  
products.



1 It is the object of the subject invention to provide a process  
for inactivating viruses in which various kinds of viruses are  
killed by the use of phenothiazine dyes without any functional-  
ly detrimental effects on the plasma proteins. It is a further  
5 object of the invention that said process be of simple design,  
such that blood or blood products may be subjected to direct  
treatment in commercially available blood bags and the added  
dyes may be removed after processing if so desired.

10 The specified object is accomplished in accordance with the in-  
vention in that the phenothiazine dyes are used at a concentra-  
tion of from 0.1 to 2  $\mu\text{M}$  and irradiation is effected directly  
in transparent containers, such as blood bags, of the kind used  
for the collection and storage of blood.

15 The irradiation is performed either with daylight of sufficient  
intensity or with monochromatic light, preferably from a cold  
light source at a wavelength in the range of the absorption  
peak of the respective dye. Also, the following conditions  
20 should be observed for virus inactivation in blood plasma or  
plasma protein solutions: The operating temperature should be  
in the range of from 0 to 37°C, if possible from 4 to 20°C.  
The inactivating time ranges especially from 5 minutes to 5  
hours, preferably from 10 minutes to 3 hours, and pH should be  
25 between pH 5 and pH 9, preferably between pH 6 and pH 8.

30 An essential advantage of the process according to the inven-  
tion lies in its simplicity. F. Heinmets et al. (as specified  
above) describes a highly complex apparatus through which, for  
instance, blood plasma must be passed. Here, problems of main-  
tenance and above all capacity arise. Surprisingly, it has now  
been found that substantially smaller quantities of dye are  
sufficient and that no complex technical apparatus is required  
for photoinactivation.

35

Unexpectedly, it has also been found that a non-enveloped vi-  
rus, such as adenovirus, which could not be inactivated under  
physiological conditions in plasma, could be photosensitized by



1 a freezing/thawing step and could thus be inactivated. In this  
connection inactivation has been ascertained irrespective of  
the employed order of the freezing/thawing steps and the addi-  
5 tion of the dye. Freezing here means a deep-freezing operation  
at temperatures of from approximately  $-20^{\circ}\text{C}$  to approximately  
80K. Normally, deep-freezing is carried out at temperatures  
below  $-30^{\circ}\text{C}$ .

10 Virus inactivation may be carried out directly in blood or  
plasma bags although these are transparent only to a limited  
extent. It is merely necessary to add the dye. Then the bag in-  
clusive of its contents is exposed to light, whereafter the re-  
spective product can be further processed.

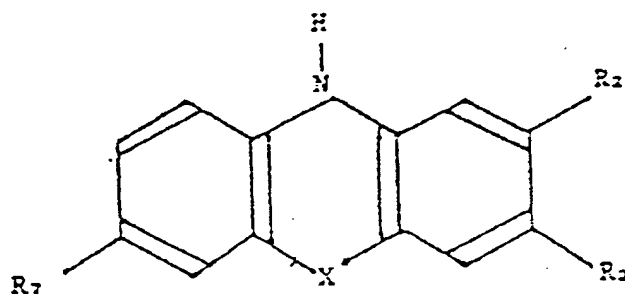
15 Thus, the process can be carried out without any major techni-  
cal effort and is excellently suited for integration in the  
processing flow of individual blood donations. The small quan-  
tity of the dye used may either remain in the treated fluid or  
may be removed by adsorbing agents.

1 Hence, the method can be carried out without any major techni-  
cal effort and is excellently suited for integration in the  
processing flow of individual blood donations. The small quan-  
tity of the dye used may either remain in the treated fluid or  
5 may be removed by adsorbing agents.

Suitable blood or blood products include:

- whole blood
- 10 - red cell concentrates
- platelet concentrates
- plasma
- serum
- cryoprecipitate
- 15 - concentrates of coagulation factors
- inhibitors
- cold insoluble globulin
- albumin.

20 Phenothiazines having the following structural formula are  
suitable for use in the method according to the invention:



	X	R <sub>2</sub>	R <sub>3</sub>	R <sub>7</sub>
neutral red	N	CH <sub>3</sub>	NH <sub>2</sub>	N(CH <sub>3</sub> ) <sub>2</sub>
35 toluidine blue	S	CH <sub>3</sub>	NH <sub>2</sub>	N(CH <sub>3</sub> ) <sub>2</sub>
methylene blue	S	H	N(CH <sub>3</sub> ) <sub>2</sub>	N(CH <sub>3</sub> ) <sub>2</sub>
phenothiazine	S	H	H	H



1

Example 1

5 Below, the dependence of photoinactivation on methylene  
blue (MB) is shown for <sup>vesicular stomatitis virus</sup> (VSV) in human plasma.

Varying concentrations of MB were added to human plasma con-  
taining approximately  $5 \times 10^7$  Plaque Forming Units (PFU) per ml  
10 of VSV. Control samples did not contain any dye. The sample  
volume was 0.5 ml. One control sample and a portion of the MB  
containing samples were irradiated with visible light for 4 h  
at room temperature; the others were stored in the dark for the  
same length of time. The light source used was a slide projec-  
15 tor equipped with a halogen bulb of 150 W (Osram Xenophot). The  
distance between the slide projector lens, i.e. the light out-  
let, and the samples was 30 cm in these and all further tests  
(with the exception of blood bag virus inactivation).

20 Following completion of irradiation, the virus titer was deter-  
mined in all samples by means of a plaque assay. The indicator  
cells used were BHK cells. The test results are listed in Ta-  
ble 1.

25

30

35

1	Samples	MB concentr. ( $\mu$ M)	Light	Virus Inactiva- tion Factor
	contr.1	0	+	4.8
5	contr.2	0	-	1
	1	0.01	+	11.8
	2	0.1	+	28.5
	3	0.5	+	$>10^6$
	4	1	+	$>10^6$
10	5	10	+	$>10^6$
	6	50	+	$>10^6$
	7	100	+	$>10^6$
	8	1	-	1
	9	10	-	5
15	10	50	-	11.8
	11	100	-	95

Table 1: Inactivation of VSV in human plasma with and without illumination.

20 Exposure time: 4 h

25 The results of Table 1 show that the infectious titer of VSV was reduced by a factor of more than  $6\log_{10}$  at a minimal MB concentration of about 0.55  $\mu$ M. Significantly higher concentrations of the dye, from about 50  $\mu$ M and up, resulted in a significant reduction in the VSV titer even without exposure to light.

30 Example 2

The following test confirmed virus inactivation at low dye concentrations.

In the presence of plasma and varying amounts of methylene blue in aliquots of 500  $\mu$ l, VSV was irradiated overnight in a cold-storage room with the slide projector from a distance of 30 cm. Samples A to F were illuminated, sample G was not.



The results of this test are presented in Table 2. They show that under the above-mentioned conditions the VSV used was inactivated by a factor of more than  $4 \log_{10}$ . This required 0.5  $\mu\text{M}$  of methylene blue.

It is probable that the VSV titer had already been reduced by 1 to 2 logs by the overnight incubation at  $4^{\circ}\text{C}$ , which would explain the relatively low initial titer. However, this was not simultaneously tested in our experiment.

A comparison of A (exposed) and G (dark) shows that light alone evidently does not influence the infectiousness of the virus to any great extent.

Sample	final MB concentr. $\mu\text{M}$	Titer/200 $\mu\text{l}$	Inactivation factor
A	0	$2 \times 10^4$	2.2
B	0.01	$2.4 \times 10^4$	1.8
C	0.05	$2 \times 10^4$	2.2
D	0.25	$3 \times 10^2$	147
E	0.5	$\leq 1$	$> 4.4 \times 10^4$
F	1.0	$\leq 1$	$> 4.4 \times 10^4$
G	0	$4.4 \times 10^4$	1

Table 2: Virus inactivation at low dye concentrations

### Example 3

The photoinactivation of viruses in the presence of phenothiazine dyes depends on the exposure time. To find out what exposure times would be sufficient for photoinactivation of VSV, 106 Plaque Forming Units (PFU) per ml were suspended in plasma and illuminated as described for different times at  $22^{\circ}\text{C}$ . The results are listed in Table 3. It is evident that under the



1 specified test conditions an exposure time of one hour was sufficient to reduce the infectious VSV titer by a factor of more than  $6\log_{10}$ .

5

Sample	Exposure time (min)	Inactivation factor
control	0	1
1	5	50
10 2	30	$1666_6$
3	60	$>10^6$

Table 3: Kinetics of the photoinactivation of VSV by MB

15

Example 4

20 A similar test was carried out in the presence of  $1 \mu\text{M}$  of another phenothiazine dye, TB, instead of MB. The results listed in Table 4 show that effective inactivation of VSV can also be achieved by using TB.

25

Sample	Exposure time (min)	Inactivation factor
control	0	1
1	10	20
2	60	$>4 \times 10^3$

30

Table 4: Kinetics of the photoinactivation of VSV by TB

35 The inactivating effect of the phenothiazine dyes was also shown for herpes simplex virus (HSV) and for type 1 human immunodeficiency virus (HIV-1).



1 Example 5

HSV is also inactivated in the presence of methylene blue  
(1  $\mu$ M). Table 5 shows the kinetics of photoinactivation of HSV  
5 by MB.

	Sample	Exposure time (min)	Inactivation factor
10	control	0	1
	1	20	35
	2	60	1500
	3	180	$>3 \times 10^4$

15 Table 5: Kinetics of the photoinactivation of  
HSV by MB

Example 6

20

A similar test was conducted with the AIDS virus HIV-1. The vi-  
rus titer was  $6 \times 10^2$  PFU/ml. MT4-cells were used as indicator  
cells. Table 6 shows that HIV-1 is apparently especially sensi-  
25 tive to photoinactivation: the virus titer was already reduced  
by a factor of more than 600 within the first 10 minutes.

25

	Sample	Exposure time (min)	Inactivation factor
30	control	0	1
	1	10	$>500$
	2	60	$>600$
	3	120	$>600$

35

Table 6: Kinetics of the photoinactivation of  
HIV-1 by MB

1 Example 7

There was no success in an attempt to inactivate non-enveloped  
viruses under the usual physiological conditions in the pres-  
5 ence of 80% plasma. As an example of a non-enveloped virus,  
adenovirus was pre-incubated for a prolonged period of time  
(4°C, dark) in the presence of methylene blue (MB) dye, 1 µM.  
Then, irradiation was effected for 30 minutes with halogen  
bulbs (150,000 lx). There was no change in the infectiousness  
10 of adenovirus.

Sample	Pre-Incubation time	Dye	Virus titer (log10)
15 control	0 h	--	6.0
1	0 h	MB	6.0
2	1 h	MB	5.5
3	4 h	MB	6.0
4	24 h	MB	6.0

20

Table 7: Influence of the pre-incubation time on the  
photosensitization of adenovirus.

The virus titer was determined as TCID50 (calculation method  
25 "Tissue Culture Infectious Dosis" by Spearman and Kaerber). The  
virus was titrated on FL cells (defined cell line suitable for  
virus titration).

When toluidine blue was used under the same experimental condi-  
30 tions, there was also no reduction of the virus titer that  
could be detected.

To achieve inactivation of adenovirus, a freeze/thaw step (F/T)  
with deep-freezing to -30°C was incorporated in the test run.  
35 Here, the order of F/T and the addition of the dye (1 µM MB)  
was of secondary importance only. The samples were again ir-  
radiated using halogen bulbs, as described above. 120,000 lx  
were measured.

1

	Sample Preparation of Sample	Virus Titer (log10)
5	control 7.5	
	A F/T	7.0
	B F/T + 60 min irradiation	7.5
	C F/T + MB + 60 min pre-incubation + 60 min irradiation	2.5
10	D MB + F/T	7.5
	E MB + F/T + 10 min irradiation	5.0
	F MB + F/T + 30 min irradiation	5.0
	G MB + F/T + 60 min irradiation	4.0

15 Table 8: Photosensitization of adenovirus due to an incorporated F/T step.  
Virus titration was carried out as described in Table 7.

20 Example 8

The special problem when using high dye concentrations is in the immediate effect of these substances on plasma proteins. Therefore, the influence of different dye concentrations on the activities of coagulation factors was investigated in a further test.

25

30

Varying amounts of MB were added to human plasma (2-ml aliquots). The activities of the coagulation factors V, VIII and IX were measured immediately thereafter. As is evident from Table 9, said factors are inhibited in all three cases in dependence on the concentration of the dye, whereby the activities of the factors VIII and V are inhibited from about 10  $\mu$ M and those of factor IX already from 2.5  $\mu$ M. Consequently, at higher concentrations MB has a direct effect on the proteins, without need of the action of light.

35



1	Methylene Blue ( $\mu\text{M/l}$ )	Factor V E/ml ;	Factor VIII E/ml	Factor IX E/ml
	0	0.80	0.38	2.0
5	1	0.76	0.41	1.9
	2.5	0.78	0.41	1.6
	5	0.74	0.38	1.45
	10	0.54	0.35	1.20
	20	0.44	0.28	1.10

10

Table 9: Influence of MB on the activities of  
coagulation factors

15

Example 9

20

However, it is not only the dye concentration used but also the exposure time which influences the activities of coagulation factors. This time-dependence has been determined for varying concentrations of methylene blue.

25

Human plasma (aliquots of 2 ml) received varying amounts of MB and was then exposed to light for 1 to 4 hours (as described in Example 1). Control samples were not subjected to photo-treatment. As is evident from Table 10, the activities of the three coagulation factors V, VIII and IX are inhibited in dependence on time and the concentration of the dye. Especially in the cases of factors VIII and IX higher MB concentrations and exposure times from 2 hours upwards cause an apparent increase in their thrombolytic activities.

30



1	Exposure time	MB Concentra- tion $\mu\text{M}/\text{l}$	Factor V E/ml	Factor VIII E/ml	Factor IX E/ml
		0	0.86	0.33	1.20
5	0 h	1	0.86	0.45	1.20
		2.5	0.82	0.33	0.46
		10	0.72	0.30	0.44
		0	0.84	0.40	0.76
10	1 h	1	0.72	0.24	0.92
		2.5	0.68	0.24	0.82
		10	0.47	0.16	0.68
		0	0.82	0.44	0.10
15	2 h	1	0.64	0.23	0.90
		2.5	0.68	0.22	0.72
		10	0.60	0.15	0.74
		0	0.76	0.38	0.98
20	4 h	1	0.55	0.16	0.94
		2.5	0.49	0.29	0.82
		10	0.42	0.27	0.64

25 Table 10: Influence of light and MB on the activities of  
coagulation factors: dependence on time and MB-concentration

30 Example 10

35 In accordance with a preferred embodiment of the subject inven-  
tion the photoinactivation of viruses may be effected directly  
in the plasma bag. The dye at the required concentration is  
merely added to the blood or the blood products and then the  
bag is exposed to light. In this simple way it is possible at  
any time to treat blood products from individual donors.



1 In a test three samples of fresh human plasma were thawed. Each  
sample was then inoculated with  $1.5 \times 10^6$  PFU VSV within the  
respective plasma bags. MB at concentrations of 1 and 10  $\mu\text{M}$ ,  
5 respectively, was added to two samples. A sample was taken from  
the MB-free plasma and stored in the dark at  $4^\circ\text{C}$  as a positive  
control. Then, the three bags were mounted between two Plexi-  
glas plates to ensure a highly uniform layer thickness of ap-  
prox. 2.5 cm. In turn, said samples were irradiated by means of  
10 a slide projector from a distance of approx. 90 cm. After 4  
hours, samples were taken to determine the virus titer and the  
same was measured by plaque assay on FL-cells. The results  
listed in Table 9 show that 1  $\mu\text{M}$  MB is already sufficient to  
reduce the infectious titer of VSV by a factor of more than  
15  $3 \log_{10}$  by means of a four-hour exposure in the plasma bag. Even  
in the absence of the dye the exposure resulted in a reduction  
of the virus titer, although only by about 50%.

Sample	Exposure time (h)	MB Concentra- tion ( $\mu\text{M}$ )	Weight of Bag (g)	VSV Titer (PFU/ml)
control	0	0	323	$5 \times 10^3$
1	4	0	323	$2.5 \times 10^3$
2	4	1	289	0
25 3	4	10	257	0

Table 11: Photoinactivation of VSV in plasma bag

30 The phenothiazine dyes used for virus inactivation may remain  
in the blood or the blood products, particularly at the concen-  
trations used here, without side effects occurring. However,  
they may be removed later by means of dialysis, gel filtration  
or adsorption.

35 Of the specified methods the adsorptive ones are of main inter-  
est because they require the least effort as to time and tech-  
nical apparatus, and the respective plasma protein solutions  
are not diluted.

However, some adsorbing agents are obviously unsuitable, such as the ion exchangers mentioned by Hiatt (Concepts in Radiation Cell Biology, pp. 57-89, Academic Press, New York, 1972) because in addition to the dye they also strongly bind plasma proteins, such as coagulation factors.

- 5 Surprisingly, it has now been found that MB and other phenothiazine dyes bind very strongly to a various commercially available separation gels, including those which either do not or only weakly bind proteins. Such adsorbing agents are therefore especially suitable for the later removal of the photo-oxidant. Of the adsorbing agents tested, the following ones may be used for the removal
- 10 of MB and other phenothiazine dyes.

Adsorbing Agent	M a t e r i a l	Manufacturer or Supplier
Daltosil 75	Modified Silica Gel	Serva, Heidelberg (FRG)
Si 100-Polyol RP 18	Derivatized Silica Gel containing C <sub>18</sub> -groups	Serva, Heidelberg (FRG)
15 Kieselgel 40	Silica Gel	Merck, Darmstadt (FRG)
Nucleosil 50 Å pore size	Silica Gel	Macherey & Nagel, Düren (FRG)
Nucleosil 100 Å pore size	Silical Gel	Macherey & Nagel, Düren (FRG)
20 Vydac SC-201 RP	Glass beads coated with Silica Gel bearing C <sub>18</sub> -groups	Macherey & Nagel, Düren (FRG)
CPG 40	Controlled pore glass (porous glass beads)	Pierce Europe (FRG)
25 Bio beads, Amberlite adsorbent resins	Polystyrene DVB (Divinylbenzene), Polyacrylester	Bio Rad, München (FRG) Röhm & Haas, Frankfurt (FRG)

- 30 In most cases 2 g of the respective adsorbing agent, used as a batch, were sufficient at a feed concentration of 10 µM to completely extract the dye from a plasma protein solution.



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Two types of adsorbing agents proved to be particularly suitable:

5

1. Silica gels having pores of so small a size (40 to approx. 100 A diameter) that plasma proteins cannot penetrate the gel matrix while the low molecular weight dye molecules can do so and are thus bonded thereto due to ionic, electrostatic and hydrophobic interaction.

10

Examples of commercially available adsorbing agents of this type are Matrex Silica Gel (Amicon, Witten), Daltosil (Serva, Heidelberg ) and Kiesel-Gel (Merck, Darmstadt).

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2. Gels of the type based on polystyrene divinyl benzene and acrylic ester polymer, respectively. They, too, are manufactured with suitable pore sizes.

20

Examples of commercially available gels of these types are Amberlite (Röhm & Haas, Frankfurt, among others) and Bio Beads (Bio Rad, München). They are mainly used to remove non-polar substances or surface-active agents such as detergents from aqueous solutions. They are either non-polar or only slightly polar.

25

Example 11

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Methylene blue (10 µM) was added to fresh plasma. 5-ml aliquots received varying amounts of Daltosil (pore size 75 A) and Bio Beads SM16 (pore size 144 A), respectively, and were then stirred for 30 minutes. Then the gel was left to settle. In the plasma the factor VIII and factor V contents, extinction at 660 nm and, for some samples, the protein contents were measured.

35



		E (660 nm)	Protein (mg/ml)	Factor VIII (U/ml)	Factor V (U/ml)
1					
	Fresh plasma	0.909	66.8	1.10	1.20
	Fresh plasma + MB	1.450	65.6	0.42	0.96
5	Daltosil 50 mg	0.576	—	0.60	1.05
	100 mg	0.571	—	1.10	1.10
	250 mg	0.491	—	1.10	1.20
	500 mg	0.477	66.8	1.25	1.20
10	Bio Beads				
	SM 16 50 mg	0.666	—	0.82	1.05
	100 mg	0.571	—	1.05	1.10
	250 mg	0.571	—	1.05	1.10
	500 mg	0.530	72.5	0.80	1.15
15					

Table 12: Extraction of methylene blue in the bag

It is evident from the extinction values that apparently further substances in addition to the dye are extracted from the plasma. But these substances are not plasma proteins. The extinction values of the plasma which had been treated with 100 to 250 mg of adsorbing agent per 5 ml, i.e. with 2 to 5 weight percent (% w/v), hardly differ from those which had been extracted with 10% w/v adsorbing agent. Hence, at an MB concentration of 10  $\mu$ M 2 to 5% w/v of adsorbing agent are sufficient in both cases for removing the dye from the plasma in a batchwise operation. If the feed concentration of the dye is lower, the amount of adsorbing agent required is correspondingly lower.

#### Example 12

In a further test a 5% human serum albumin solution (5% HSA) was used instead of blood plasma. Again, the MB concentration was 10  $\mu$ M. Aliquots of 5 ml were extracted batchwise with 100 mg, respectively, i.e. 2% w/v, of the following adsorbing



1 agents for varying periods of time: Daltosil (pore size 75 A  
Kiesel-Gel (pore size 40 A) and Bio Beads SM16 (pore size  
144 A).

5 As Figure 1 shows, the extinction at 660 nm decreases to a con-  
stant value in all three cases within a period of 20 to 30 min-  
utes, i.e. this time period is sufficient to remove the photo-  
oxidant in batches from a plasma protein solution. As is fur-  
ther evident from Figure 1, Bio Beads SM16 and Kiesel-Gel 40  
10 appear to be somewhat better adsorbing agents in the subject  
case than Dalto sil with a pore size of 75 A.

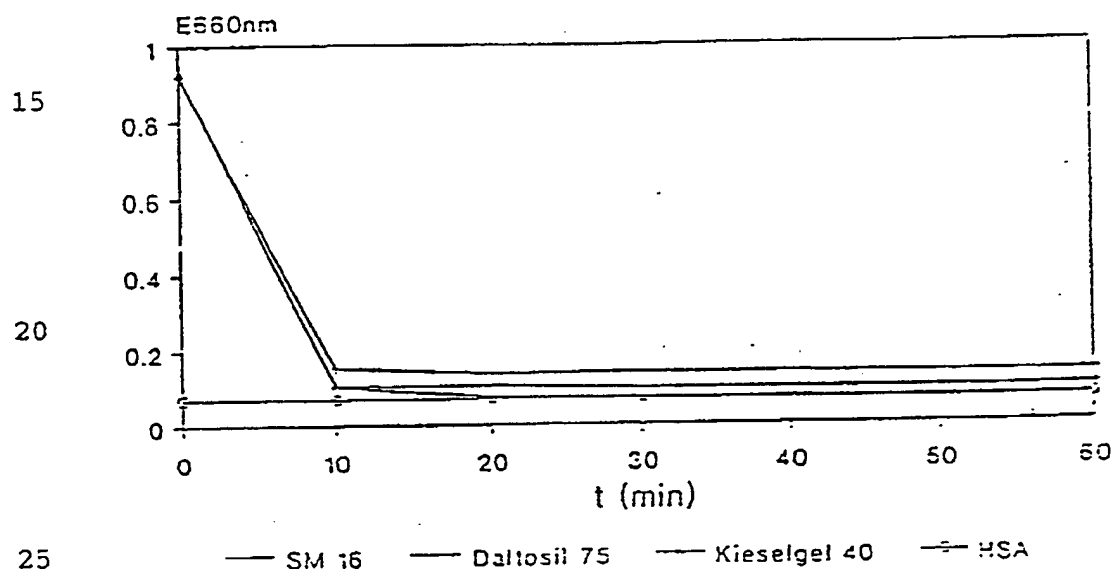


Fig. 1: Adsorbition kinetics of methylene blue (10 µM) at RT  
with HSA 5% 100 mg gel/5 ml HSA



Example 13


Removal of MB from plasma protein solutions by column chromatography

The aim of this test was to find out whether or not the adsorptive removal of the photo-oxidant can also be effected by chromatography. This was based on the idea of carrying out the virus inactivation by means of a dye in combination with light in a container, such as a blood bag, and in turn transferring the plasma protein solution to another container, such as a second blood bag, via a small separating column interposed between said containers, and containing the adsorbing agent. If the assembly, comprising the first bag, the adsorbing column and the second bag were prefabricated so that a closed system were available, it would be possible in a very simple way and at the minimum risk of contamination to produce virus-inactivated plasma protein preparations, including from single donor units.

To this end 250 ml of 5% albumin solution were passed at varying flow rates through a separating column containing 5 ml of Kiesel-Gel (pore size 40 A). Fractions of 10 ml each were collected and their extinction was measured at 660 nm.

As can be seen from Table 13, the overall volume of the albumin solution could be passed through the column at flow rates of 5 and 7.5 ml/min, respectively, and no MB residues could be detected in the fractions coming off the column. Hence, the time required for removal of the dye from 250 ml of solution is only 30 to 35 minutes at most.

The test result shows that the removal of the photo-oxidant by chromatography may be effected without any problems, and also proves that the above-mentioned production of virus-inactivated plasma protein preparations from single donor units is indeed possible.



1	Starting Material + MB		Flow Rate (ml/min)	
			5	7.5
	extinction (660 mμ): 0.067		extinction	(660 mμ)
	fraction No. 1		0.002	0.001
5	3		0.000	0.001
	5		0.000	0.002
	7		0.002	0.003
	9		0.001	0.001
	11		0.000	0.001
10	13		0.000	0.001
	14		0.002	0.001

Table 13: Chromatographic separation of MB from a 5% albumin solution (1 μM MB concentration)

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PCT/DE90/00691

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PROCESS FOR INACTIVATING VIRUSES IN BLOOD  
AND BLOOD PRODUCTS

## Claims:

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1. A process for inactivating viruses in blood and blood products, comprising: adding phenothiazine dyes to the solutions or suspensions to be treated and subsequently irradiating said phenothiazine-dye containing solutions or suspensions with visible light in the range of the absorption peak of the respective dye, whereafter the blood or blood products may be passed over adsorbing agents for removal of the dyes, characterized in that the phenothiazine dyes are used at a concentration of from 0.1 to 2  $\mu\text{M}$  and irradiation is effected directly in transparent containers, such as blood bags, used for collecting and storing blood.

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2. The process as claimed in claim 1, characterized in that toluidine blue or methylene blue is used as the phenothiazine dye.

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3. The process as claimed in any one of the claims 1 or 2, characterized in that the solutions or suspensions to be treated are initially subjected to deep-freezing and are then thawed prior to irradiation.

4. The process as claimed in claim 3, characterized in that the dye is added prior to the deep-freezing step.

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5. The process as claimed in claim 3, characterized in that the dye is added after thawing and prior to irradiation.



1 6. The process as claimed in any one of the claims 1 to 5,  
characterized in that said process is carried out using two  
containers suitable for collecting blood, such as blood bags,  
5 with a separating column interposed between said containers,  
and containing the adsorbing agent for the phenothiazine dyes.

7. The process as claimed in claim 6, characterized in that the  
adsorbing agents used are silica gels or such agents based on  
polystyrene divinylbenzene or acrylic ester polymers.

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# INTERNATIONAL SEARCH REPORT


International Application No. PCT/DE 90/00691

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (In several classification symbols apply, indicate all:*)				
According to International Patent Classification (IPC) or to both National Classification and IPC				
Int.Cl. <sup>5</sup> : A 01 N 1/02				
<b>II. FIELDS SEARCHED</b>				
Minimum Documentation Searched				
Classification System:	Classification Symbols:			
Int.Cl. <sup>5</sup> :	A 01 N; C 12 N			
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *				
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b>				
Category *	Citation of Document: ** with indication, where appropriate, of the relevant passages	* Relevant to Claim No. **		
X	Proceedings of the Society for Experimental Biology and Medicine, vol. 148, 1975, pages 291-293, Te-Wen Chang et al.: "Photodynamic Inactivation of Herpes-virus Hominis by Methylene Blue (38524)", see the whole document	1-10		
X	Concepts in Radiation Cell Biology, chapter 2, 1972, pages 57-89, C.W. Hiatt, "Methods for Photo-inactivation of Viruses", see pages 79-83	1-10		
X	Photochemistry and Photobiology, vol. 29, 1979 Wallace Snipes et al.: "Inactivation of lipid-containing viruses by hydrophobic photosensitizers and near-ultraviolet radiation", see page 785 - page 790 and the whole document	1-10		
X	J. gen. Virol., vol. 41, 1978 Grace S.L. Yen et al.: "Photosensitization of Herpes Simplex Virus Type 1 with Neutral Red", see page 273 - page 281 and the whole document	1-10		
P, X	WO, A1, 9007876 (NEW YORK UNIVERSITY) 26 July 1990, see claims 11-13: page 3, lines 20-23	1		
<p>* Special categories of cited documents:</p> <table style="width: 100%;"> <tr> <td style="width: 50%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p> </td> </tr> </table>			<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p>
<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p>			
<b>IV. CERTIFICATION</b>				
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report		
29 November 1990 (29.11.90)		19 December 1990 (19.12.90)		
International Searching Authority:		Signature of Authorized Officer:		
European Patent Office				

III. EINSCHLAGIGE VERÖFFENTLICHUNGEN (Fortsetzung von Blatt 2)		
Art	Kennzeichnung der Veröffentlichung, soweit erforderlich unter Angabe der maßgeblichen Teile	Betr. Anspruch Nr.
X	J. gen. Virol., Band. 41, 1978 Grace S.L. Yen et al.: "Photosensitization of Herpes Simplex Virus Type 1 with Neutral Red", siehe Seite 273 - Seite 281 und das ganze Dokument  --	1-10
P,X	WO, A1, 9007876 (NEW YORK UNIVERSITY) 26 Juli 1990, Siehe Ansprüche 11-13; Seite 3, Zeilen 20-23  -- -----	1

# INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen PCT/DE 90/00691

<b>I. KLASSIFIKATION DES ANMELDUNGSGENSTANDS</b> (bei mehreren Klassifikationssymbolen sind alle anzugeben) <sup>6</sup>		
Nach der Internationalen Patentklassifikation (IPC) oder nach der nationalen Klassifikation und der IPC		
InLCl.5 A 01 N 1/02		
<b>II. RECHERCHIERTE SACHGEBIETE</b>		
Recherchierter Mindestprüfstoff <sup>7</sup>		
Klassifikationssystem	Klassifikationssymbole	
InLCl.5	A 01 N; C 12 N	
Recherchierte nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Sachgebiete fallen <sup>8</sup>		
<b>III. EINSCHLÄGIGE VERÖFFENTLICHUNGEN<sup>9</sup></b>		
Art *	Kennzeichnung der Veröffentlichung <sup>11</sup> , soweit erforderlich unter Angabe der maßgeblichen Teile <sup>12</sup>	Betr. Anspruch Nr. <sup>13</sup>
X	Proceedings of the Society for Experimental Biology and Medicine, Band 148, 1975, Seiten 291-293, Te-Wen Chang et al: "Photodynamic Inactivation of Herpesvirus Hominis by Methylene Blue (38524)", Siehe das ganze Dokument	1-10
X	Concepts in Radiation Cell Biology, Kapitel 2, 1972, Seiten 57-89, C.W. Hiatt, "Methods for Photo-inactivation of Viruses", Siehe Seiten 79-83	1-10
X	Photochemistry and Photobiology, Band. 29, 1979 Wallace Snipes et al.: "Inactivation of lipid-containing viruses by hydrophobic photosensitizers and near-ultraviolet radiation", siehe Seite 785 - Seite 790 und das ganze Dokument	1-10
<p>* Besondere Kategorien von angegebenen Veröffentlichungen<sup>10</sup>:</p> <p>"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist</p> <p>"E" Älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist</p> <p>"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)</p> <p>"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht</p> <p>"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist</p> <p>"T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist</p> <p>"X" Veröffentlichung von besonderer Bedeutung, die beanspruchte Erfindung kann nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden</p> <p>"Y" Veröffentlichung von besonderer Bedeutung, die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist</p> <p>"Z" Veröffentlichung, die Mitglied derselben Patentfamilie ist</p>		
<b>IV. BESCHEINIGUNG</b>		
Datum des Abschlusses der internationalen Recherche	Absenddatum des internationalen Recherchenberichts	
29. November 1990	19. 12. 90	
Internationale Recherchenbehörde	Unterschrift des bevollmächtigten Bediensteten	
Europäisches Patentamt	F.W. HECK 	

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. PCT/DE 90/00691

SA 39764

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on 01/11/90  
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9007876	26/07/90	AU-D- 5085190	13/08/90

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

ANHANG ZUM INTERNATIONALEN RECHERCHENBERICHT  
ÜBER DIE INTERNATIONALE PATENTANMELDUNG NR. PCT/DE 90/00691

SA 39764

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patendokumente angegeben.

Die Angaben über die Familienmitglieder entsprechen dem Stand der Datei des Europäischen Patentamts am 01/11/90.

Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

Im Recherchenbericht angeführtes Patendokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
WO-A1- 9007876	26/07/90	AU-D- 5085190	13/08/90

Für nähere Einzelheiten zu diesem Anhang : siehe Amtsblatt des Europäischen Patentamts, Nr.12/82